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Research Article

# Which species of water frogs inhabit ponds of the Caucasus? Taxonomic mess with *Pelophylax ridibundus* species complex

David Tarkhnishvili[1], Mariami Todua[1], Giorgi Iankoshvili[1]

1 *Institute of Ecology, Ilia State University, Tbilisi, Georgia*
*Corresponding author: David Tarkhnishvili (david_tarkhnishvili@iliauni.edu.ge)*

## Abstract

Two nominal species of water frogs of the genus Pelophylax, P. ridibundus and P. bedriagae, are found in Central and Eastern Europe, the Middle East, Western Asia, Western Kazakhstan, and Siberia. So far, the taxonomic status of Pelophylax in most of the Caucasus has remained unknown. Sequencing of the Cytochrome Oxidase 1 mitochondrial gene attributed the frogs throughout Georgia to the P. 'bedriagae' lineage, bringing them very close to the specimens from Kazakhstan and Greece. Simultaneously, the current nomenclature of water frogs appears to be formally incorrect since western Kazakhstan, the type locality for P. ridibundus, has frogs genetically closer to nominal P. bedriagae, than to nominal P. ridibundus from Europe. Because there is no evidence that the frogs from Central Europe, Kazakhstan, and the Caucasus, as well as from Anatolia and Iran, are biological species with individual evolutionary pathways, we suggest a conservative approach and synonymize nominal P. bedriagae from most of West Asia with P. ridibundus.

**Key words:** mitochondrial DNA, *Pelophylax ridibundus*, *Pelophylax bedriagae*, water frog taxonomy

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## Introduction

During most of the XX century, the Western Palaearctic water frogs were attributed to two species: small-bodied *Rana lessonae* from Western Europe and large-bodied *Rana ridibunda* from Central and Eastern Europe, Central and Western Asia, and Northern Africa; the hybrid form between *R. ridibunda* and *R. lessonae*, *R. esculenta*, was originally described as a separate species (Hellmich 1962; Bannikov et al. 1977; Kuzmin 1999). Based on the analysis of enzyme profiles, Berger (1983) suggested the presence of three other species of the group, *R. perezi* from the Iberian Peninsula and two species with a limited distribution – from Italy and Greece. The taxonomic revolution exploded in the early 1990s. First, the polyphyletic origin of the nominal genus *Rana* was shown (Wiens et al. 2009), and Palaearctic water frogs were attributed to the genus name *Pelophylax*; hence, *R. ridibunda* was renamed *Pelophylax ridibundus*. Then, the nominal *P. ridibundus* has been split into several geographically distinct taxa. Schneider et al. (1992) suggested a species status for frogs from West Asia and named it *Pelophylax bedriagae* (see also Sinsch and Schneider 1999). Plötner et al. (2001) suggested the presence of two additional cryptic

species in Anatolia and the Middle East, *P. caralitanus* and *P. cypriensis*, although the former one was recently synonymized with *P. bedriagae* (Sinsch et al. 2023). Later, a few other species were described, including *R. saharica*, synonymized with *R. perezi* (Steinwarz and Schneider 1991; see the recent discussion in Dufresnes et al. (2024)).

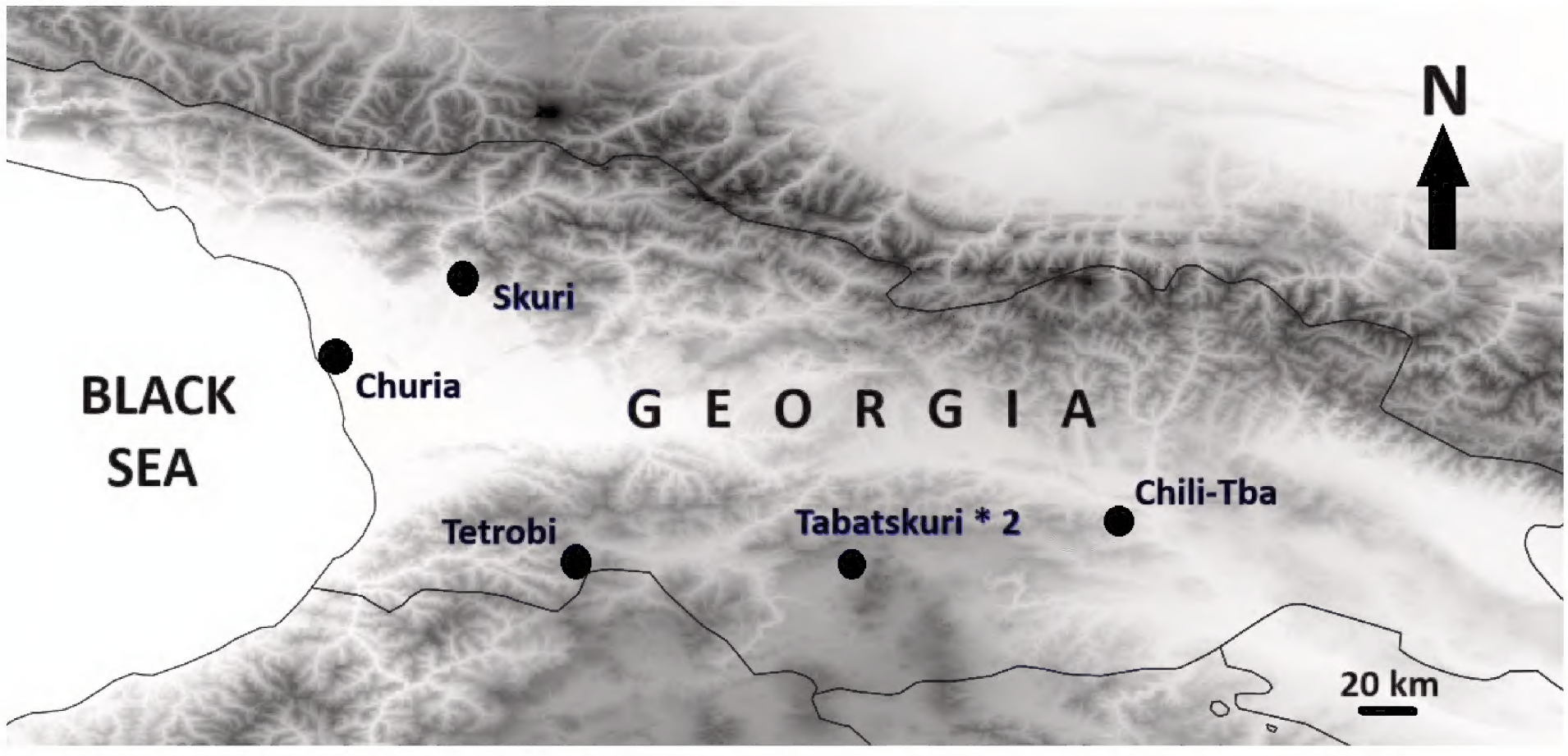
## Materials and methods

Samples (toe phalanges) of frogs were collected from five Georgian locations (Fig. 1), representing different parts of the country, from both the Black and Caspian Sea basins and the Greater and Lesser Caucasus Mountain systems. Tissue samples were stored in 95% alcohol for further DNA extraction.

### DNA analyzes

DNA purification was processed using the Quick-DNA Miniprep Plus Kit (Zymo Research). Partial sequences of cytochrome oxidase subunit I (COI) were amplified by polymerase chain reaction (PCR) using the primer pairs LCOI490-JJ and HCO2198-JJ (Astrin and Stüben 2008). Thermal conditions included denaturation at 95 °C for 1 min, followed by the first cycle set (15 cycles): 94 °C for 30 s, annealing at 55 °C for 1 min (–1 °C per cycle), and extension at 72 °C for 1:30 min. Second cycle set (25 cycles): 94 °C for 35 s, 45 °C for 1 min, 72 °C for 1:30 min, followed by 1 cycle at 72 °C for 3 min, and the final extension step at 72 °C for 5 min.

PCR amplicons were visualized on 1% agarose gels using 1.7 µl of PCR product. The unpurified PCR products were sequenced in both directions at the Beijing Genomics Institute (Hong Kong, CN) using the amplification primers. Sequence analysis was performed using Geneious Prime 2022.1.1 (http://www.geneious.com). Extracted DNA was deposited in the scientific collections of Ilia State University, Tbilisi, Georgia, and aliquots will be deposited at the LIB




**Figures 1.** Sampling locations of *Pelophylax* sp. in Georgia.

Biobank at Museum Koenig, Bonn, Germany. At the same time, the sequences have been submitted to Barcode of Life Data System (BOLD) databases. The newly obtained DNA barcodes of COI sequences were checked out against the BOLD systems and BLAST database (http://www.boldsystems.org/, https://blast.ncbi.nlm.nih.gov/Blast.cgi).

In the analysis, we used six obtained CO1 mitochondrial gene sequences of *Pelophylax* from throughout Georgia and 89 sequences of water frogs downloaded from NCBI GenBank (Benson et al. 2012) from Turkey, Kazakhstan, the Balkan Peninsula, Central, and Western Europe that belong to the nominal species *Pelophylax ridibundus*, *P. bedriagae*, *P. kurtmuelleri*, *P. caralitanus*, *P. perezi*, *P. lessonae*, *P. mongolius*, and a few non-identified *Pelophylax*. The access numbers, origin of the samples, and nominal species names are shown in the Suppl. material 1.

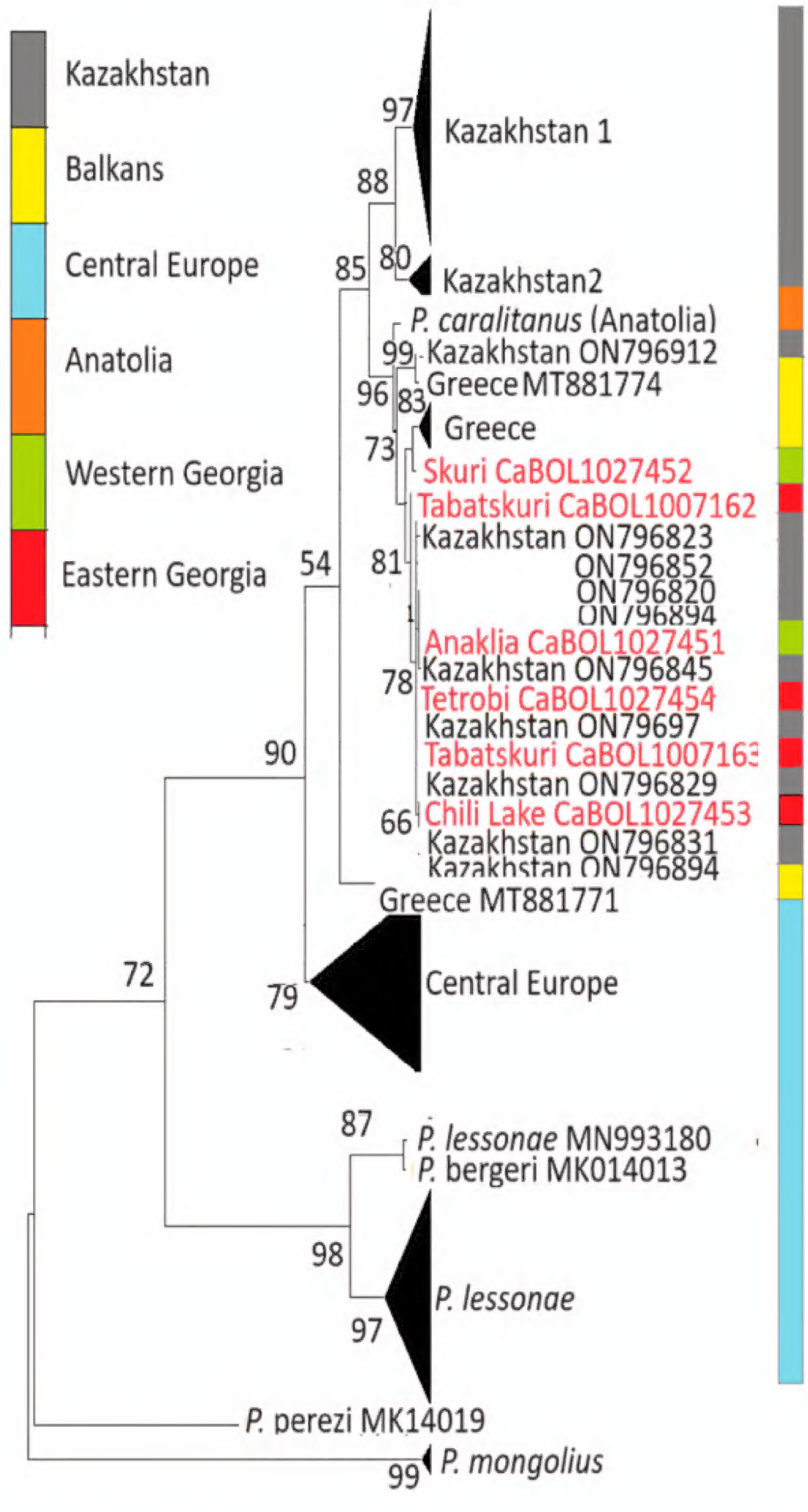
## Results

Pairwise genetic distances between Georgian samples and green frogs from throughout West Eurasia, based on the analysis of Cytochrome Oxidase 1 sequences (COI), are shown in Suppl. material 2. The distances between the 545 bp long fragment of COI of samples from Georgia and Kazakhstan vary between 0–4% (3.4%), between samples from Georgia and Greece – between 1-5.5% (2.0%), between Georgia and Central Europe (excluding *P. lessonae*, but including nominal *P. kurtmuelleri*) – 5-6% (5.7%) and between Georgian samples and *P. caralitanus* – 1-1.5%. The differences between the nominal *P. ridibundus* and *P. bedriagae* on one side and *P. lessonae* – 14-15%, between the nominal *R. ridibundus* and *R. perezi* – 15 %. In general, sequence differences between geographic populations of the nominal *P. ridibundus* + *P. bedriagae* groups vary between 0-6% and are proportional to the geographic distance between the populations. In contrast, sequence divergence between this group and *P. lessonae*, as well as *P. perezi* and *P. mongolius*, is qualitatively higher and usually exceeds 14%.

The Maximum Likelihood tree of the obtained haplotypes is shown in Fig. 2. The Georgian mitochondrial sequences of the nominal *P. ridibundus* belong to a clade shared with the nominal *P. ridibundus* from Kazakhstan and the nominal *P. bedriagae* from Greece. The differences between the haplotypes of frogs from Georgia are minor but still exceed those between some individuals from Georgia and Kazakhstan and Georgia and Greece. In general, based on the similarity of mitochondrial haplotypes, there is no evidence of the non-conspecific status of frogs from the entire Caucasus, west and north of the Caspian Sea Basin, Anatolia, and the Balkan Peninsula.

## Discussion

Several publications tried to infer the phylogeny of *Pelophylax*, based on the analysis of mitochondrial genes (Plötner et al. 2001; Lymberakis et al. 2007; Akin et al. 2010; Hofman et al. 2016; Dufresnes et al. 2017, 2024; Ualiyeva et al. 2022; Papežík et al. 2023). There is a consensus suggesting monophyly of a widespread subclade including nominal *P. ridibundus*, *P. bedriagae*, *P. kurtmuelleri*, *P. caralitanus*, *P. cerigensis*, and *P. cypriensis* (although the separate




**Figures 2.** Maximum Likelihood tree of water frogs from Europe and West Asia (see also Suppl. material 1), including six samples from Georgia. Bootstrap values are shown at the nodes.

species status of *P. caralitanus* and *P. cerigensis* is not supported by genetic data – see Limberakis et al. (2007), Akin et al. (2010), Sinsch et al. (2023) and Dufresnes et al. (2024)). The phylogenetic pattern within this subclade depends on the sampling areas. Dufresnes et al. (2017) suggested that *P. bedriagae* and *P. ridibundus*/*P. kurtmuelleri* are sister, reciprocally monophyletic taxa. Their results are in line with studies by Lymberakis et al. (2007) and Papežík et al. (2023), although the latter authors suggested the ingroup status for *P.*

*cypriensis* within this subclade. However, these findings contradict the results of Plötner et al. (2001) and Ualiyeva et al. (2022), who rejected the reciprocal monophyly of *P. ridibundus* and *P. bedriagae*.

The situation with these two species remained unclear. There are no fixed morphological characters that could distinguish between *P. ridibundus* and *P. bedriagae* (Sinsch and Schneider 1999), but there are obvious differences between mating calls of water frogs from Europe and the Middle East, including southern Anatolia (Schneider et al. 1992). Mating call analysis of frogs from Armenia and Kazakhstan placed them closer to European *P. ridibundus* than to Middle Eastern *P. bedriagae* (Schneider and Sinsch 1999). The mitochondrial haplogroup of the frogs from the Levant does not cluster with those from most of Anatolia, and it has a sister status to the clade found throughout Europe, most of Anatolia, and the Caspian area (Plötner et al. 2001; Ualiyeva et al. 2022).

Simultaneously, the latter clade contains two reciprocally monophyletic haplogroups, one from the Balkans, Anatolia, the Caucasus, and Western Kazakhstan, and another one from Central Europe. As Schneider and Sinsch (1999) fairly stated, frogs from Western Kazakhstan are undoubtedly *P. ridibundus*, considering that this is Terra Typica for this species and mating calls of the frogs from this area do not differ from those of *P. ridibundus* from Central Europe, and the same applies to the frogs from Armenia. However, later researchers routinely attributed frogs from the Balkans and Western Asia to *P. bedriagae*. Plötner et al. (2001) used the name *P. bedriagae* for all frogs from Western Asia. Lymberakis et al. (2007) classified all frogs from the southern Balkans, Anatolia, and the Middle East as *P. bedriagae*. Ualiyeva et al. (2022) attributed the mitochondrial haplotypes of water frogs from western Kazakhstan to *P. bedriagae* because they were clustered with the sequences from Anatolia (but not with *P. bedriagae* from the Levant) and not with the haplogroup of *P. ridibundus* from Central Europe. Dufresnes et al. (2024) also used the name *P. bedriagae* for frogs from Central Asia.

Our results support the reciprocal monophyly of mitochondrial haplogroups of marsh frogs from (1) the Balkans, the Caucasus, Western and Central Asia, and (2) Central Europe. However, we suggest that the name *Pelophylax bedriagae* can only be applied to the frogs from the Levant, which Schneider et al. described as a separate species back in 1992. Applying this name to the frogs from most of Anatolia, the Caucasus, and Central Asia, including Kazakhstan, is incorrect because (a) western Kazakhstan, and specifically the Atyrau district, is Terra Typica for *P. ridibundus* (Pallas 1771). By definition, they should be attributed to *P. ridibundus*, assuming that all marsh frogs from this area are conspecific; (b) the vocalization of frogs from western Kazakhstan, Central Europe, and the Caucasus (Armenia) is similar as opposed to the frogs from the Middle East (Schneiderand Sinsch 1999). The frogs from the northern (Ermakov et al. 2016) and the southern (this paper) Caucasus, the region that is on the way between Anatolia and the Caspian Area/Kazakhstan, also have the haplogroup widespread in the Balkans and Anatolia.

Ualiyeva et al. (2022) showed that the nuclear gene SAI of frogs from the *P. ridibundus* group is separated into three haplogroups. They did not show reciprocal monophyly between nominal *P. ridibundus* and *P. bedriagae*. In the Terra typica of *P. ridibundus*, two nuclear haplogroups are present, one closer to the Anatolian and the other to the Central European frogs. Finally, a genetic study

of invasive marsh frogs in Belgium showed introgressive hybridization between nominal *P. ridibundus* and *P. 'bedriagae'*, suggesting the absence of reproductive barriers between these two (Holsbeek et al. 2009).

In conclusion, the presence of distinct mitochondrial haplogroups of marsh frogs from the north-west and south-east of their western Eurasian range reflects a long period of isolation between the European and West Asian or Balkan populations. However, it is not sufficient to attribute them to different species. There are no vocalization differences between the frogs from Kazakhstan, the Caucasus, and Central Europe, and no data is available for those Anatolian populations, which belong to the mitochondrial lineage widespread in Western Asia and the Caucasus. Nuclear haplotypes more common in Central Europe and Anatolia are admixed in the Terra Typica of *P. ridibundus* and are occasionally found in the same population of Western Kazakhstan; finally, there is no evidence of reproductive isolation between these populations. The proportion of the sequence differences cannot be taken as evidence of the effective isolation of "different evolutionary pathways" (in the sense of DeQueiroz 2007). There is no evidence of a limited gene flow or the presence of tension zones between the eastern and western geographic populations or marsh frogs. While reserving the name *R. bedriagae* for frogs from some populations from the Levant and southern Anatolia, marsh frogs from most of Anatolia, the Balkans, the Caucasus, and the Caspian area should all be attributed to the species *P. ridibundus*.

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## Additional information

### Conflict of interest

The authors have declared that no competing interests exist.

### Ethical statement

No ethical statement was reported.

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### Author contributions

GI planned field work and collected samples, MT performed genetic analysis and tree building, DT wrote the text with assistance of GI and MT, all authors participated in final data analysis.

### Author ORCIDs

David Tarkhnishvili https://orcid.org/0000-0003-1479-9880
Mariami Todua https://orcid.org/0000-0003-1870-3030
Giorgi Iankoshvili https://orcid.org/0000-0002-0429-1488

## Data availability

All of the data that support the findings of this study are available in the main text or Supplementary Information.

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## Supplementary material 1

### Samples and downloaded sequences used in the study

Authors: Tarkhnishvili D, Todua M, Iankoshvili G

Data type: GenBank identification numbers

Explanation note: Samples and downloaded sequences (NCBI identification numbers) used in the study.

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# Supplementary material 2

## Genetic distances (proportion of differences) between the studied sequences

Authors: Tarkhnishvili D, Todua M, Iankoshvili G

Data type: Genetic distance matrix

Explanation note: Genetic distances (proportion of differences) between the studied sequences of CO1 mitochondrial gene of *Pelophylax*.

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